

Phylogenetic Study of Sumatran *Impatiens* (Balsaminaceae) Using Nuclear and Plastid DNA Sequences

NANDA UTAMI* AND MARLINA ARDIYANI

*Molecular Systematics Laboratory, Herbarium Bogoriense, Research Center for Biology-LIPI
Cibinong Science Center, Jl Raya Jakarta-Bogor Km.46, Cibinong 16911, Indonesia.*

**utami_16002@yahoo.com (author for correspondence)*

Although there have been several studies on the phylogeny of *Impatiens*, no such studies have focused on *Impatiens* in Sumatra. This study aimed to reveal the phylogeny of Sumatran *Impatiens* and evaluate their position among species from other regions. The *atpB-rbcL* intergenic spacer from plastid DNA and the Internal Transcribed Spacer region from nuclear ribosomal DNA (1414 bp in total) was sequenced for 24 samples representing 18 species of *Impatiens*, including 16 species endemic to Sumatra. Parsimony analyses were done with the addition of outgroups and sequences of *Impatiens* from the NCBI GenBank. According to the strict consensus of 2120 most parsimonious trees from the combined data set, *Impatiens* is monophyletic with 93% bootstrap support. The ITS and *atpB-rbcL* data showed that Sumatran *Impatiens* is distributed in more than one clade depicting multiple origins from southern China. The addition of Sumatra-endemic species indicates that *Impatiens* probably originated in southern China.

Key words: *atpB-rbcL*, *Balsaminaceae*, biogeography, endemic, *Impatiens*, ITS

The Balsaminaceae, a family consisting mostly of succulent and subsucculent annual or perennial herbs, are usually regarded as comprising two genera, the monotypic *Hydrocera* and the prolific *Impatiens* with an estimated 850 species mainly distributed in montane areas of the Old World tropics and subtropics (Grey-Wilson 1980, Yuan *et al.* 2004). High diversity is in tropical Africa, Madagascar, southern India, the eastern Himalaya, Southeast Asia, and southwest China (Grey-Wilson 1980). *Impatiens* is represented on almost all the islands in the Malesian region, especially in the Philippines, Borneo, Java, Sumatra and New Guinea (Grey-Wilson 1989). *Impatiens* in the mountainous regions of Sumatra, the Bukit Barisan range is particularly diverse. Since Grey-Wilson's treatment in 1989, several additional species of *Impatiens* have been described

from there (Shimizu & Utami 1997, Utami 2005, 2009, 2011, 2012a, 2012b).

Impatiens suffers from a rather confused taxonomy and an absence of recent comprehensive revisions. The first and last attempt to treat the entire genus was made by Warburg and Reiche (1895), who classified the genus into two subgenera and 14 sections based on leaves, flowers and type of spur. More recent revisions or studies have been on a regional scale such as in Africa, Sumatra (Grey-Wilson 1980) and China (Grey-Wilson 1980, Yu 2012) and in Thailand (Shimizu 1977).

Although there have been several recent studies of *Impatiens*, few have included Sumatran species. Fujihashi *et al.* (2002) presented a molecular phylogeny of *Impatiens* based on the *rbcL* and *trnL-trnF* spacer. Yuan *et al.* (2004) utilized

Internal Transcribed Spacer (ITS) sequences to study intrageneric relationships in Balsaminaceae and Janssens *et al.* (2006, 2007, 2009, 2010, 2011) studied *Impatiens* using both chloroplast and nuclear sequences. Lens *et al.* (2012) compared the phylogeny of Balsaminaceae using wood anatomy and *atpB-rbcL* spacer and nuclear ImpDEF1/ImpDEF2 sequences. We used a molecular approach, utilizing nuclear ITS and chloroplast *atpB-rbcL* sequence data, to determine the relationships of the endemic species of *Impatiens* in Sumatra.

Materials and Methods

DNA of the plastid region of the *atpB-rbcL* intergenic spacer and the nuclear region of ITS were sequenced for 18 species representing 16 endemic species of *Impatiens* from Sumatra and 2 species from Java and Sulawesi (Table 1). Ac-

cording to the results of Yuan *et al.* (2004) and Janssens *et al.* (2006), *Norantea guianensis*, *Hydrocera triflora*, and an unknown species of *Souroubea* were selected as outgroups. DNA sequences from another 30 species of *Impatiens*, obtained from the NCBI GenBank (Table 2), representing various geographical areas were added to the analysis. These made a total of 57 accessions for the analysis. All new sequences have been deposited in the NCBI GenBank database.

Total genomic DNAs were extracted from silica gel dried leaves by the modified cetyltrimethyl ammonium bromide (CTAB) method of Doyle & Doyle (1987). Nuclear DNA sequences of Internal Transcribed Spacer (ITS) and chloroplast DNA sequences of the intergenic spacer (IGS) between *atpB* and *rbcL* were used in this study. The *atpB-rbcL* and ITS regions were amplified using KAPA2G Robust PCR Kits (KAPA Biosystems Inc., Boston, US) with a programmable

TABLE 1. Species of *Impatiens* used in this study.

| No | Species | Locality | Voucher | Acc. no. (ITS) | Acc. no. (<i>atpB-rbcL</i>) |
|----|---|---------------------------------|---------------|-------------------|----------------------------------|
| 1 | <i>I. alboflava</i> Miq. | Paninggahan, West Sumatra | NU 2046 (BO) | KJ472431 | KJ472455 |
| 2 | <i>I. batanggadensis</i> Utami acc.1 | Batang Gadis, North Sumatra | NU 2043A (BO) | KJ472432 | KJ472456 |
| 3 | <i>I. batanggadensis</i> Utami acc.2 | Batang Gadis, North Sumatra | NU 2043B (BO) | KJ472433 | KJ472457 |
| 4 | <i>I. beccarii</i> Hook. f. ex Dunn. | Solok, West Sumatra | NU 2051 (BO) | KJ472434 | KJ472458 |
| 5 | <i>I. chonoceras</i> Hassk. | Java (common) | NU 2054 (BO) | KJ472435 | KJ472459 |
| 6 | <i>I. diepenhorstii</i> Miq. acc.1 | Air Sirah, West Sumatra | NU 2044A (BO) | KJ472436 | KJ472460 |
| 7 | <i>I. diepenhorstii</i> Miq. acc.2 | Mt. Singgalang, West Sumatra | NU 2044B (BO) | KJ472437 | KJ472461 |
| 8 | <i>I. ekapaksiana</i> Utami acc.1 | Surian, West Sumatra | DG 1536A (BO) | KJ472438 | KJ472462 |
| 9 | <i>I. ekapaksiana</i> Utami acc.2 | Surian, West Sumatra | DG 1536B (BO) | KJ472439 | KJ472463 |
| 10 | <i>I. elephanticeps</i> Grey-Wilson | Kerinci Seblat NP, West Sumatra | NU 2056 (BO) | KJ472440 | KJ472464 |
| 11 | <i>I. junghuhnii</i> Miq. | Mt. Sinabung, North Sumatra | NU 2049 (BO) | KJ472441 | KJ472465 |
| 12 | <i>I. kerinciensis</i> Utami | Kerinci Seblat NP, West Sumatra | WR 347 (BO) | KJ472442 | KJ472466 |
| 13 | <i>I. kunyitensis</i> Utami | Kerinci Seblat NP, West Sumatra | WR 341 (BO) | KJ472443 | KJ472467 |
| 14 | <i>I. phyrrhotrica</i> Miq. | Ladang Padi, West Sumatra | NU 2050 (BO) | KJ472444 | KJ472468 |
| 15 | <i>I. pilosivittata</i> Grey-Wilson | Mt. Singgalang, West Sumatra | NU 2052 (BO) | KJ472445 | KJ472469 |
| 16 | <i>I. platypetala</i> Lindl. acc.1 | South Sulawesi (common) | NU 2053 (BO) | KJ472446 | KJ472470 |
| 17 | <i>I. platypetala</i> Lindl. acc.2 | South Sulawesi (common) | NU 2057 (BO) | KJ472447 | KJ472471 |
| 18 | <i>I. platypetala</i> Lindl. acc.3 | Sulawesi (common) | MA 155 (BO) | KJ472448 | KJ472472 |
| 19 | <i>I. rubricaulis</i> Utami | West Sumatra | R&N 487 (BO) | KJ472449 | KJ472473 |
| 20 | <i>I. sidikalangensis</i> Grey-Wilson acc.1 | Sidikalang, North Sumatra | NU 2048A (BO) | KJ472450 | KJ472474 |
| 21 | <i>I. sidikalangensis</i> Grey-Wilson acc.2 | Sidikalang, North Sumatra | NU 2048B (BO) | KJ472451 | KJ472475 |
| 22 | <i>I. tapanuliensis</i> Grey-Wilson | Mt. Sinabung, North Sumatra | NU 2055 (BO) | KJ472453 | KJ472477 |
| 23 | <i>I. tribuana</i> Utami | Solok Ambah, West Sumatra | DG 1534 (BO) | KJ472454 | KJ472478 |
| 24 | <i>I. sp.</i> | Gunung Kopi, Aceh, Sumatra | NU 2058 (BO) | KJ472452 | KJ472476 |

TABLE 2. Species of *Impatiens* and outgroups obtained from NCBI GenBank.

| No | Species | Distribution | Acc. no. (ITS) | Acc. no. (<i>atpB-rbcL</i>) |
|----|----------------------------|-------------------------|-------------------|----------------------------------|
| 1 | <i>I. auricoma</i> | Madagascar | AY348748 | DQ147815 |
| 2 | <i>I. balsamina</i> | southern India, SE Asia | AY348749 | DQ147816 |
| 3 | <i>I. campanulata</i> | southern India | AY348758 | DQ147822 |
| 4 | <i>I. capensis</i> | North America | AY348759 | DQ147823 |
| 5 | <i>I. chinensis</i> | SE Asia | AY348761 | DQ147825 |
| 6 | <i>I. columbaria</i> | West Africa | AY348764 | DQ147828 |
| 7 | <i>I. conchibracteata</i> | southern China | AY348765 | DQ147829 |
| 8 | <i>I. congolensis</i> | Central Africa | AY348766 | DQ147830 |
| 9 | <i>I. fenghwaiana</i> | southern China | AY348779 | DQ147842 |
| 10 | <i>I. fischeri</i> | East Africa | AY348781 | DQ147843 |
| 11 | <i>I. flanaganianae</i> | East Africa | AY348783 | DQ147846 |
| 12 | <i>I. forrestii</i> | southern China | AY348784 | DQ147847 |
| 13 | <i>I. glandulifera</i> | Himalaya | AY348788 | DQ147848 |
| 14 | <i>I. hians</i> | West Africa | AY348791 | DQ147849 |
| 15 | <i>I. kerrii</i> | Southeast Asia | AY348799 | DQ147853 |
| 16 | <i>I. kilimanjari</i> | Africa | AY348800 | FJ826656 |
| 17 | <i>I. niamniamensis</i> | Central Africa | AY348812 | DQ147862 |
| 18 | <i>I. omeiana</i> | southern China | HQ718765 | DQ147864 |
| 19 | <i>I. parviflora</i> | Europe, northern Asia | AY348816 | DQ147866 |
| 20 | <i>I. repens</i> | southern India | AY348826 | DQ147875 |
| 21 | <i>I. rubrostriata</i> | southern China | AY348828 | DQ147876 |
| 22 | <i>I. soulieana</i> | southern China | AY348833 | DQ147880 |
| 23 | <i>I. stenosepala</i> | southern China | AY348835 | DQ147881 |
| 24 | <i>I. trichosepala</i> | southern China | AY348852 | DQ147885 |
| 25 | <i>I. tuberosa</i> | Madagascar | AY348844 | DQ147886 |
| 26 | <i>I. uliginosa</i> | southern China | AY348845 | DQ147887 |
| 27 | <i>I. usambarensis</i> | East Africa | AY348847 | DQ147889 |
| 28 | <i>I. viscida</i> | southern India | KF719166 | DQ147891 |
| 29 | <i>I. walleriana</i> | East Africa | AY348852 | DQ147892 |
| 30 | <i>I. yingjiangensis</i> | southern China | AY348851 | DQ147894 |
| 31 | <i>Hydrocera triflora</i> | Sri Lanka | AY348853 | DQ147895 |
| 32 | <i>Norantea guianensis</i> | | AY348855 | DQ147898 |
| 33 | <i>Souroubea</i> sp. | | AY348857 | DQ147900 |

temperature-control system PCR Thermal Cycler TaKaRa Dice. ITS amplification used primers and cycling conditions as described in Yuan *et al.* (2004). The primers used were Y4 (5'-CCC-GCCTGACCTGGGGTCGC-3') and Y5 (5'-TAGAGGAAGGAGAAGTCGTAAC-3'). The PCR profile was as follows: a 30 cycle reaction with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min, in addition to an initial denaturation at 94°C for 3 min and final extension at 72°C for 5 min. We used primers and cycling conditions described in

Janssens *et al.* 2006 to amplify the IGS between *atpB-rbcL*. The primers used were IMP-*atpB* (5'-ACATCTAGTACCGGACCAATGA-3') and IMP-*rbcL* (5'-AACACCAGCTTTGAATCCAA-3'). The PCR profile was as follows: a 30 cycle reaction with denaturation at 94°C for 1 min, annealing at 53°C for 0.5 min and extension at 72°C for 0.5 min, in addition to an initial denaturation at 94°C for 2 min, and extension at 72°C for 7 min. Purification of the PCR product as well as sequencing were done at 1stBASE (www. base-asia. com).

Phylogenetic analyses

Newly generated sequences of the ITS and the IGS between *atpB-rbcL* were contig using ChromasPro version 1.5 (Technelysium) then added to the data matrix extracted from GenBank, which included representatives of *Impatiens*, *Hydrocera triflora*, *Norantea guianensis* and a species of *Souroubea*. The data matrix was realigned using MUSCLE (Edgar 2004) as implemented in MEGA v5.10 (Tamura *et al.* 2012) followed by minor manual adjustment. Only the base substitutions were treated as characters for phylogenetic reconstruction. All base substitutions were equally weighted. The gaps were treated as missing values. The Maximum Parsimony method for phylogeny reconstruction was employed using PAUP* version 4.0 beta 10 (Swofford 2002). Heuristic search option of random addition sequence (100 replications), tree bisection and reconnection (TBR) swapping algorithm and multiple parsimonious trees (MULPARS), including uninformative characters, were used to find the most parsimonious trees. The consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated with PAUP*. Bootstrap analysis with 1000 replications (heuristic; 100 random; TBR) was performed using PAUP*.

Congruence among the data sets of the nuclear ITS and plastid *atpB-rbcL* were examined with the incongruence length difference (ILD) test (Farris *et al.* 1994), implemented in PAUP, as the partition homogeneity test. The tests were performed with 100 replications of heuristic search with random addition sequence (100 replications), TBR swapping, and MULPARS.

Results and Discussion

DNA sequence variation and Phylogenetic analysis

In *Impatiens*, the plastid *atpB-rbcL* intergenic spacers ranged from 696 to 757 bp. The nuclear ITS regions ranged from 548 to 623 bp. All OTUs analyzed in this study were combined into the same data set. Of the 897 bp of *atpB-rbcL* IGS sequences that were aligned for the analysis, 232 bp (26%) were variable, and 132 bp (15%) were

phylogenetically informative. Of the 723 bp of aligned ITS sequences, 457 bp (63%) were variable, and 346 bp (48%) were phylogenetically informative.

Parsimony analysis of the non-gapcoded data matrix of the plastid *atpB-rbcL* intergenic spacer sequences resulted in 358 equally most parsimonious trees. The CI was 0.771, the RI 0.845, the RC 0.651 and the HI 0.229. The strict consensus tree was well resolved (Fig. 2), especially among the earliest diversified branches. Bootstrap analysis showed relatively high support for many of the lineages. The MP analysis of the *atpB-rbcL* data highly supported the monophyly of *Impatiens* (BS 97%). With respect to the outgroup chosen, the *atpB-rbcL* data confirmed the sistergroup relationship between *Impatiens* and *Hydrocera*, but with low support (<50%). To compare the results, the clades in the following text and figures are named following Janssens *et al.* (2006). In the *atpB-rbcL* tree, Clades 1-15 of Janssens *et al.* (2006) were monophyletic or composed of a single OTU except for Clade 8. As in Janssens *et al.* (2006), *I. omeiana* (Clade 1) is sister to all other species of *Impatiens*. Clades 2, 3 and 4 comprised a monophyletic group with BS 89%. These clades cover southern China, Southeast Asia, the Himalaya, Europe, and northern Asia. Two Sumatran species (*I. tribuana* and *I. alboflava*) are nested in Clade 2. Clades 5 to 15 (BS 90%) and 8 to 15 (BS 72%) comprise monophyletic groups. Within the latter, the Sumatran species (except for *I. tribuana* and *I. alboflava*) form a monophyletic group with two species from southern China and a species from Java.

Parsimony analysis of the non-gapcoded data matrix of the ITS data resulted in 1716 equally most parsimonious trees. The CI was 0.498, the RI 0.635, the RC 0.316, and the HI 0.502. In the strict consensus tree (Fig. 3), *Impatiens* is not monophyletic because *Hydrocera* is nested within it, but the bootstrap support is weak (51%). A clade of *Norantea guianensis* and *Souroubea* was supported by BS 100%, whereas that of *I. alboflava* from Sumatra and *I. kerriae* from Southeast Asia was supported by BS 73%. There are other differences from the *atpB-rbcL* tree, but Clades 5

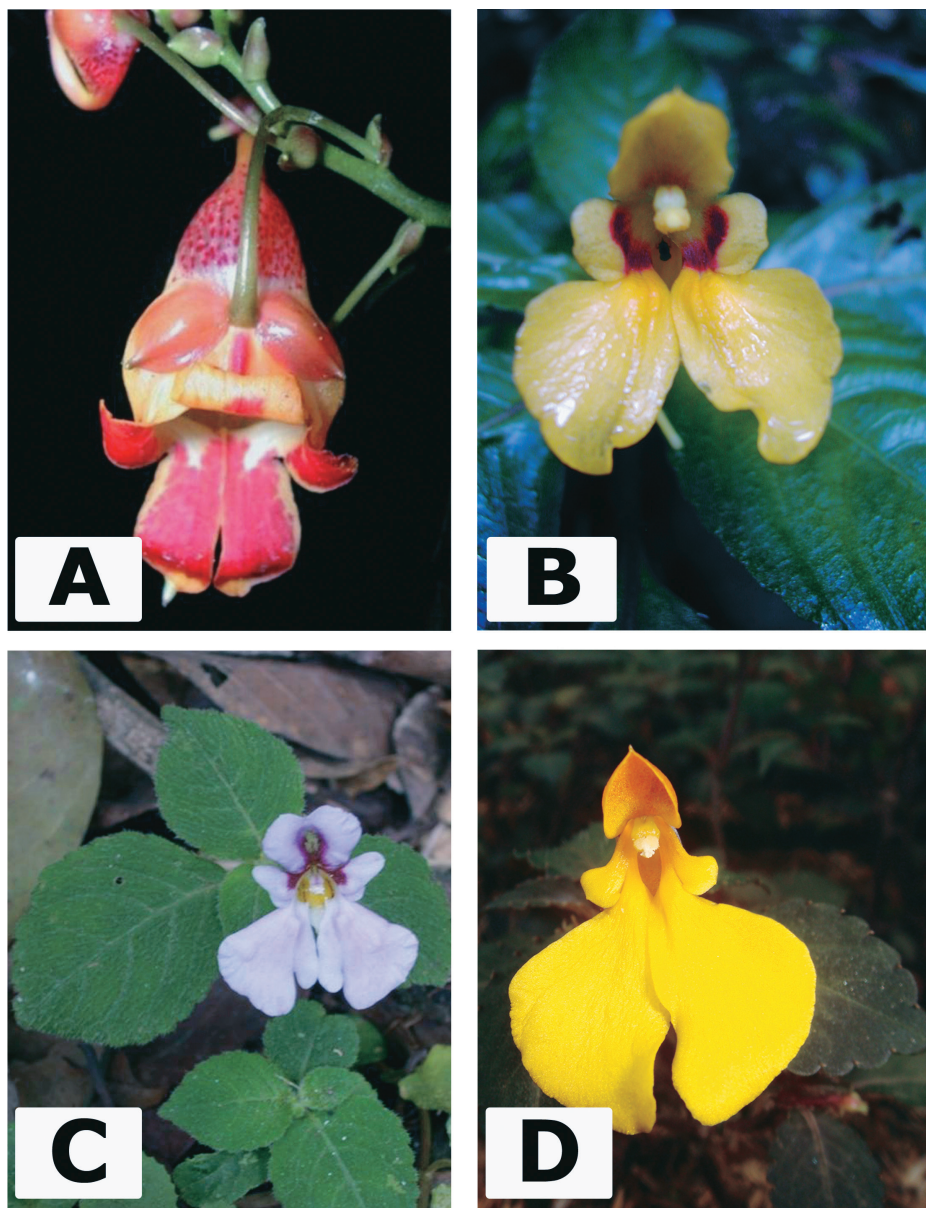


FIG. 1. *Impatiens* in Sumatra shows tremendous diversity in floral color and morphology.

A. *Impatiens tribuana*; B. *I. beccarii*; C. *I. sidikalangensis*; D. *I. rubricaulis*
(Photos by: D. Girmansyah & M. Ardiyani).

to 15 (BS 63%) and 8 to 15 (BS 98%) comprise monophyletic groups, although *Impatiens. repens* (Clade 15) is excluded from the monophyletic group of Clades 8 to 15.

The partition-homogeneity test among the data sets of the plastid *atpB-rbcL* intergenic spacer and the nuclear ITS revealed a P value of 0.01.

We concluded that the data set of the plastid *atpB*, *rbcL* intergenic spacer and the nuclear ITS data set were combinable. We therefore discuss the phylogenetic and geographic relationship of Sumatran *Impatiens* based on the topology of the combined tree. The combined analysis of the plastid *atpB-rbcL* intergenic spacer and the nu-

clear ITS sequences resulted in 2120 equally most parsimonious trees. The CI was 0.533, the RI 0.658, the RC 0.350, and the HI 0.467.

According to strict consensus (Fig. 4) of the combined data set, *Impatiens* is monophyletic with 93% bootstrap support. The topology is mostly in agreement with the consensus tree produced by the chloroplast analysis (Fig. 2) with stronger bootstrap values in many cases. Clades 2 to 7 contain the same species as in the tree resulting from the *atpB-rbcL* data. When we compared *atpB-rbcL* with the combined ITS and *atpB-rbcL* dataset, we noticed a small number of incongruent relationships between both phylogenies. The largest dissimilarities are found in the position of *I. repens*, *I. balsamina*, *I. viscida*, *I. campanulata*, *I. hians*, and *I. columbaria* which are strongly influenced by the nuclear data. The pattern of the branches is more similar to those from the ITS data. For example, according to the chloroplast data, *I. repens* and *I. balsamina*, which are both from southern India, are sisters to each other, but in the combined analysis, *I. repens* is far from *I. balsamina* and is nested between Clades 6 and 7 from southern China and North America. These conflicts might be due to the different mode of inheritance between nuclear and chloroplast DNA, but the differences need to be resolved by the analysis of additional markers before any conclusions, such as hybrid origin, can be made.

Phylogenetic position and geographic origin of Sumatran Impatiens

Except for *Impatiens beccarii*, the species of *Impatiens* in Sumatra are distributed in two clades (Fig. 4). The first, Clade 2, includes *I. tribuana* (Fig. 1A) and *I. alboflava* (BS 83%). The second, Clade 9 (BS 100%), includes the remaining species including *I. sidikalangensis* (Fig. 1C) and *I. rubricaulis* (Fig. 1D). *Impatiens beccarii* (Fig. 1B) is included in Clade 9 in the *atpB-rbcL* tree, but is remotely placed in the ITS and combined trees. The molecular phylogeny showed that the species of *Impatiens* in Sumatra originated at least twice. Clade 2 comprises two Sumatran species, *I. stenosepala*, also in southern China, and *I. kerriae*, also in Southeast Asia. *Impa-*

tiens alboflava and *I. tribuana* probably originated in southern China. Clade 9 comprises 13 species in Sumatra, 2 also in southern China and 1 in Java. An origin in southern China is suggested, probably followed by rapid diversification in the Sumatra region. Our findings suggest that the species of *Impatiens* in Sumatra are set through repetitive speciation of ancestral species from southern China. The conclusion of Janssens *et al.* (2006) that *Impatiens* probably originated in southern China is clearly supported when the endemic Sumatran species are added.

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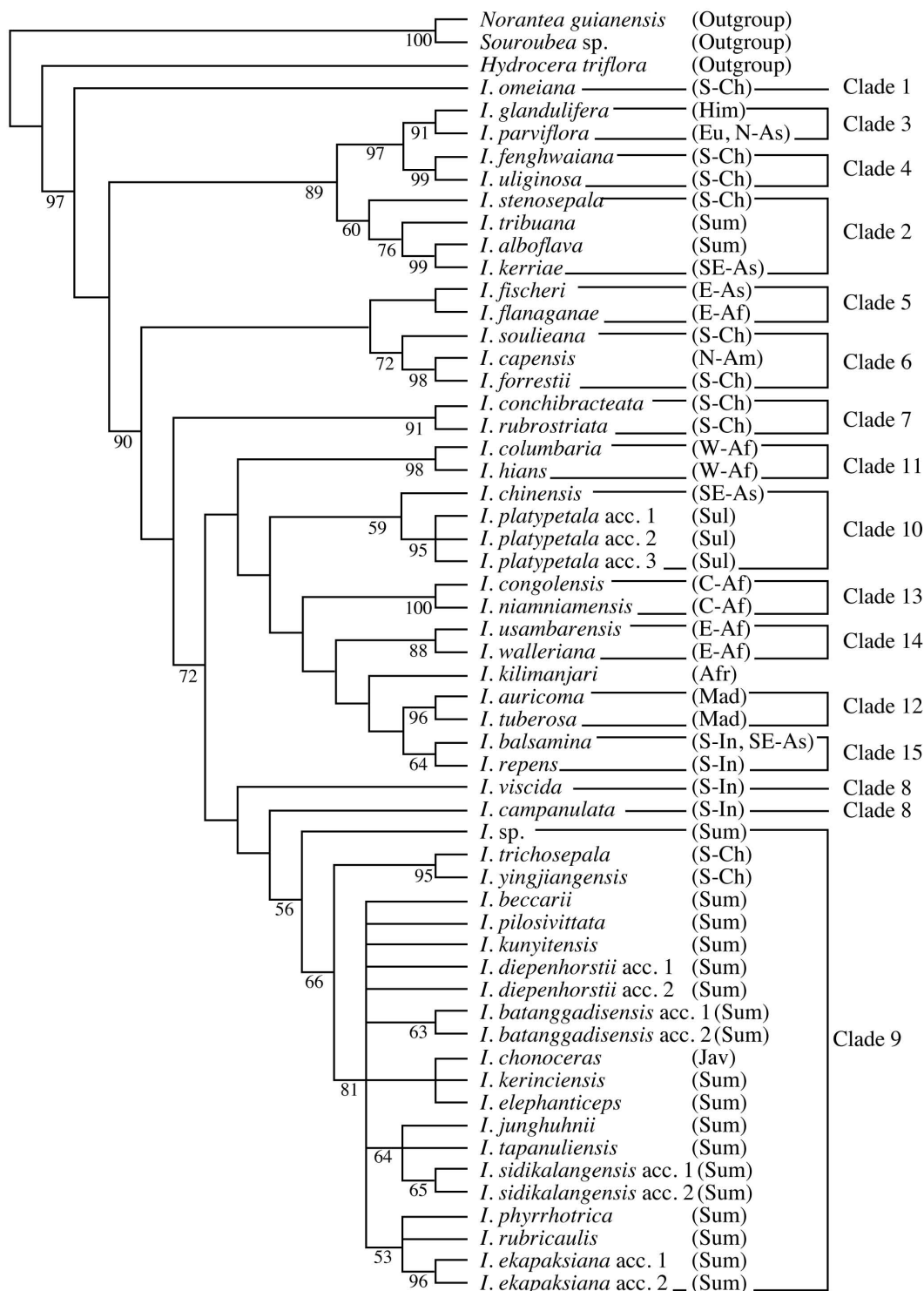


FIG. 2. Strict consensus of the 358 equally most parsimonious trees from MP analyses of IGS between *atpB-rbcL* data set. Numbers below branches are bootstrap values supporting corresponding branch when greater than 50%. Clades are numbered 1 through 15 following Janssens *et al.* (2006). Distribution area is shown after species name (C-Af: Central Africa, E-Af: East Africa, W-Af: West Africa, Afr: Africa, Mad: Madagascar, S-Ch: southern China, SE-As: Southeast Asia, N-As: northern Asia, E-As: eastern Asia, S-In: southern India, N-Am: North America, Him: Himalaya, Eu: Europe, Sul: Sulawesi, Sum: Sumatra).

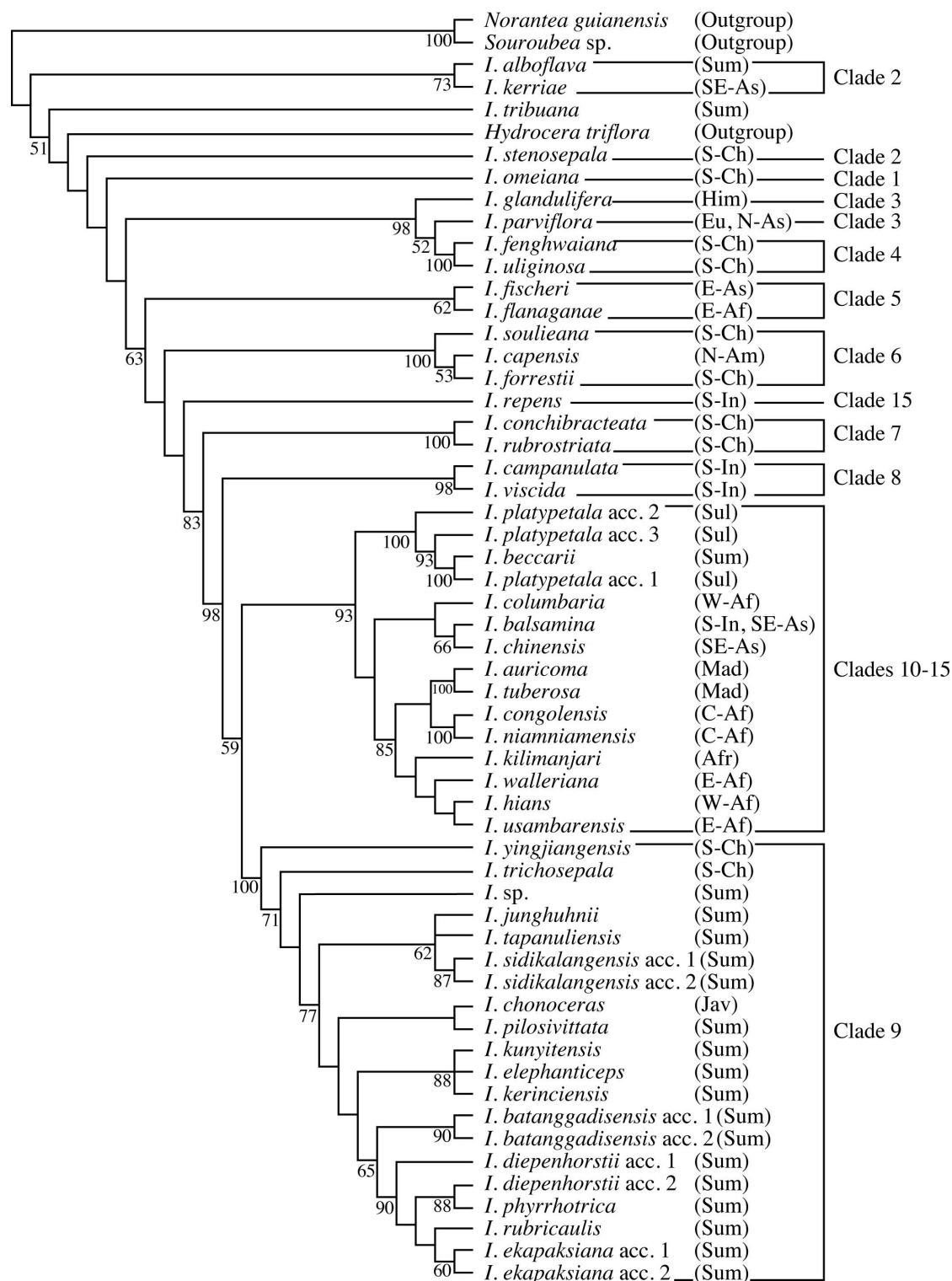


FIG. 3. Strict consensus of the 1716 equally most parsimonious trees resulting from MP analyses on the complete ITS data set. Numbers below the branches are bootstrap values supporting the corresponding branch when greater than 50%. Clades are numbered 1 through 15 following Janssens *et al.* (2006). Distribution area is shown after species name (C-Af: Central Africa, E-Af: East Africa, W-Af: West Africa, Afr: Africa, Mad: Madagascar, S-Ch: southern China, SE-As: Southeast Asia, N-As: North Asia, E-As: eastern Asia, S-In: southern India, N-Am: North America, Him: Himalaya, Eu: Europe, Sul: Sulawesi, Sum: Sumatra).

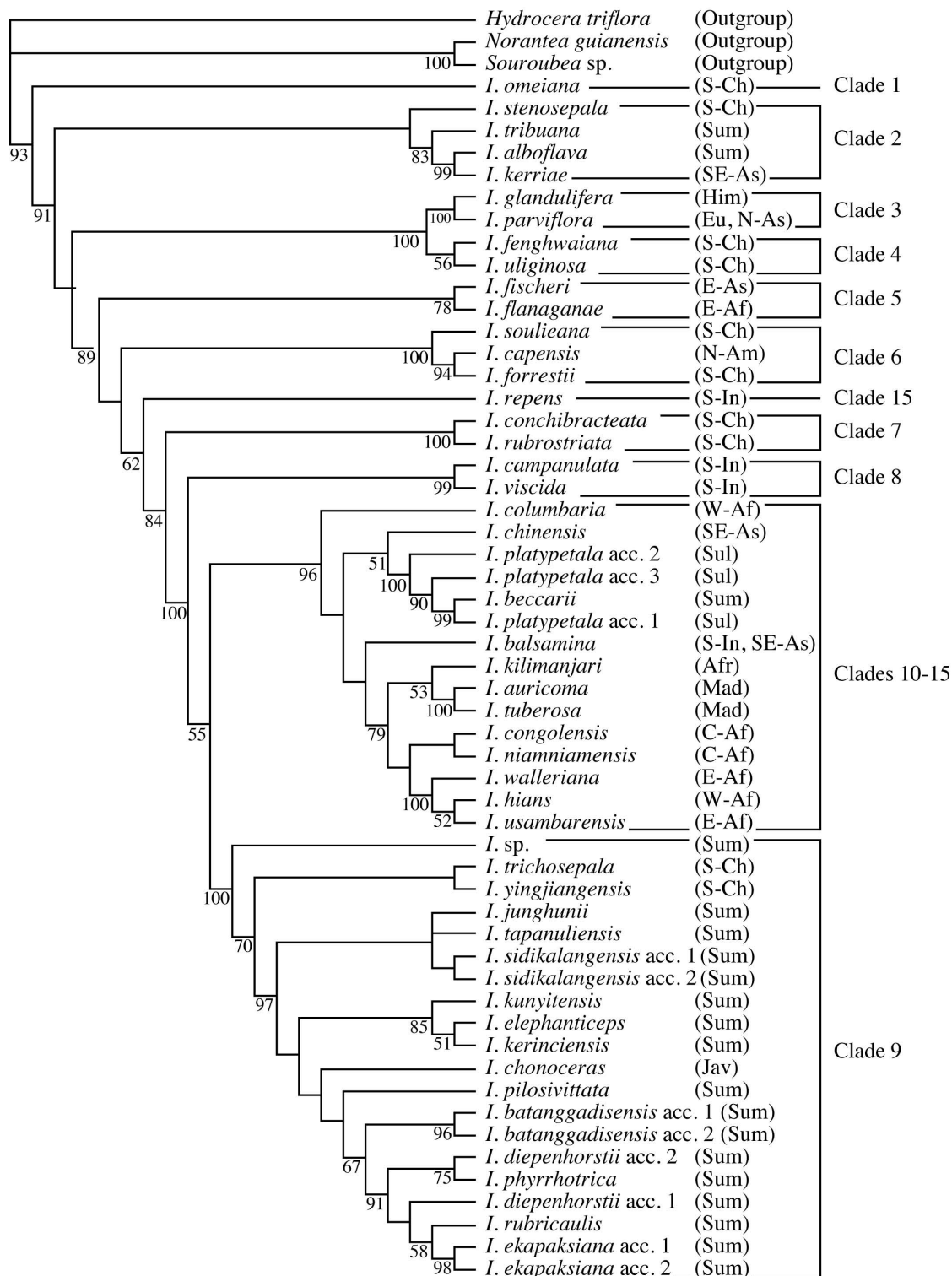


FIG. 4. Strict consensus of the 2120 equally most parsimonious trees from MP analyses of combined data sets of ITS and IGS between *atpB-rbcL* intergenic spacer. Numbers below branches are bootstrap values supporting corresponding branch when greater than 50%. Clades are numbered 1 through 15 following Janssens *et al.* (2006). Distribution area is shown after species name (C-Af: Central Africa, E-Af: East Africa, W-Af: West Africa, Afr: Africa, Mad: Madagascar, S-Ch: southern China, SE-As: Southeast Asia, N-As: northern Asia, E-As: eastern Asia, S-In: southern India, N-Am: North America, Him: Himalaya, Eu: Europe, Sul: Sulawesi, Sum: Sumatra).

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